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**Betaproteobacteria dominance and diversity shifts in the bacterial community of a
PAH-contaminated soil exposed to phenanthrene**

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25 **Abstract**

26 In this study, the PAH-degrading bacteria of a constructed wetland collecting road runoff has
27 been studied through DNA stable isotope probing. Microcosms were spiked with ¹³C-
28 phenanthrene at 34 or 337 ppm, and bacterial diversity was monitored over a 14-day period.
29 At 337 ppm, PAH degraders became dominated after 5 days by Betaproteobacteria, including
30 novel *Acidovorax*, *Rhodoferax* and *Hydrogenophaga* members, and unknown bacteria related
31 to Rhodocyclaceae. The prevalence of Betaproteobacteria was further demonstrated by
32 phylum-specific quantitative PCR, and was correlated with a burst of phenanthrene
33 mineralization. Striking shifts in the population of degraders were observed after most of the
34 phenanthrene had been removed. Soil exposed to 34 ppm phenanthrene showed a similar
35 population of degraders, albeit only after 14 days. Our results indicate that specific
36 Betaproteobacteria are involved in the main response to soil PAH contamination, and
37 illustrate the potential of SIP approaches to identify soil PAH degraders.

38 *Key words* : 16S rRNA sequences; stable isotope probing; PAH degradation; phenanthrene;
39 Betaproteobacteria

40

41

42 Capsule : On a site collecting road runoff, implementation of stable isotope probing to
43 identify soil bacteria responsible for phenanthrene degradation, led to the discovery of new
44 Betaproteobacteria distantly related to known PAH degraders.

45

46 **Introduction**

47 Among the environmental pollutants that perturb ecosystems and threaten human beings,
48 polycyclic aromatic hydrocarbons (PAHs) are of special concern because they are persistent
49 and accumulate along the trophic chain. To clean up contaminated sites, bioremediation
50 strategies have been proposed based on the ability of particular microorganisms to degrade
51 PAHs (Doyle et al., 2008). Numerous bacterial isolates able to utilize PAHs as carbon sources
52 have been described, and the biochemical pathways responsible for their oxidative
53 degradation have been investigated (Penget al., 2008). Based on their frequent occurrence on
54 polluted sites and their metabolic potential, members of the Sphingomonadaceae and
55 Actinobacteria are considered potent PAH degraders in soil or sediments (Leyset al., 2004;
56 Leyset al., 2005; Alonso-Gutierrez et al., 2009). However, in the last decade, culture-
57 independent studies highlighted the great bacterial diversity in environmental ecosystems
58 such as soils, and showed that culturable bacteria represented less than 5 % of the existing
59 species (Cole et al., 2010). Hence, PAH-degrading strains described so far might not be
60 representative of soil bacteria that actually remove pollutants in situ. Consistent with this idea,
61 investigations involving a selective labeling of the bacteria of interest through stable-isotope
62 probing (SIP (Radajewski et al., 2000)) led to the discovery of new bacteria involved in
63 pollutant degradation (Wackett, 2004). SIP-based methods permit the exploration of
64 uncultured bacteria present in natural or contaminated environments and reduce biases
65 associated with selection in artificial media (Dumont and Murrell, 2005). Using a field-based
66 SIP strategy to track naphthalene degraders on a coal tar contaminated site, Jeon *et al.*
67 identified a novel *Polaromonas* strain, as the major players (Jeon et al., 2003). In studies
68 targeting naphthalene-, phenanthrene- or pyrene-degrading bacteria in a bioreactor treating
69 PAH-contaminated soil, different bacterial taxa were detected depending on the PAH-
70 substrate used as probe (Singleton et al., 2005; Singleton et al., 2006). An *Acidovorax* strain,

71 which was found to be dominant among phenanthrene-degrading bacteria, was later isolated
72 and characterized as a novel PAH degrader (Singleton et al., 2009). Recently, bacteria related
73 to the *Pseudoxanthomonas* and *Microbacterium* genera were identified as the main
74 phenanthrene degraders in soil but diversity changes were observed in soil also treated with
75 root exudates (Cebron et al., 2011). In another SIP-based study targeting anthracene-degrading
76 bacteria, dominant soil degraders were found to be affiliated to the *Sphingomonadales* and
77 *Variovorax* taxa (Jones et al., 2011).

78 In the present study, we have implemented a SIP strategy to explore the diversity of a PAH-
79 degrading community in a constructed wetland collecting the road runoff from a highway.
80 Using ^{13}C -phenanthrene as tracer, we examined changes in the soil PAH-degrader population
81 as a function of the dose and time of exposure to the tracer. For this purpose, soil DNA was
82 extracted after various SIP treatments, separated by isopycnic centrifugation, and labeled
83 DNA were used to identify phenanthrene degraders based on 16S rRNA gene sequences. .
84 Moreover, dose- and time-dependent changes in the composition of the phenanthrene-exposed
85 bacterial community were monitored by a combination of molecular methods, including
86 single-strand conformation polymorphism and quantitative PCR. Results revealed that, in
87 addition to *Burkholderiales*, bacteria related to the Rhodocyclaceae and *Thiobacillus* taxa
88 appeared as new potential phenanthrene degraders. Our data also provide new insights into
89 the response of a large panel of specific degraders, mostly undescribed, to soil PAH
90 contamination.

91

92 MATERIALS AND METHODS

93

94 Sampling site and determination of soil PAH concentration

95 Soil samples were obtained from a constructed wetland collecting road runoffs from a
96 highway near Chambéry (France) in October 2008. The facility, administered by the AREA
97 company, was colonized by *Typha latifolia* and *Phragmites australis* all over the structure.
98 Soil was sampled from the 10-cm upper layer, sieved to about 3 mm and stored in a closed
99 plastic box at 4°C until use. The physico-chemical composition of the soil used will be given
100 elsewhere.

101

102 Synthesis of [U-¹³C]-phenanthrene

103 [U-¹³C]-phenanthrene was prepared from [U-¹³C] succinic anhydride and [U-¹³C]
104 naphthalene (both from Sigma-Aldrich), according to a previously described method
105 (Singleton, et al., 2005) and references cited therein) with minor modifications. The product
106 was obtained in five steps with a yield of approximately 44%. The purity and homogeneity of
107 the final preparation were checked by ¹H NMR in CDCl₃ (Fig. S1) and GC-MS (m/z = 192
108 (M⁺)). A detailed description of the preparation procedure is available upon request.

109

110 Microcosm setup and incubation conditions

111 Incubations were performed in 250 ml sterilized glass Erlenmeyer flasks, closed with rubber
112 stoppers. Microcosms consisted of 20 g wet soil (water content, 59 %, vol/wt) and 5 ml of a
113 salt solution (7.5 mM (NH₄)₂SO₄ – 20 mM KH₂PO₄ – 30.6 mM Na₂HPO₄ – 0.18 mM CaCl₂ –
114 3.6 10⁻² mM FeSO₄ – 0.81 mM MgSO₄), added to favor bacterial metabolism. [U-¹³C]-
115 phenanthrene was supplied as a 54.9 mM stock solution in dimethylsulfoxide (DMSO) to give
116 final concentrations of either 33.7 or 337 ppm (on a dry wt basis), equivalent to levels 200- or

2000-fold as high as that of phenanthrene initially found in soil. Relevant microcosm experiments were referred to as SIP200 and SIP2000, respectively. Two microcosms supplied with 337 ppm labeled phenanthrene were inoculated with approx. 2×10^8 cells (0.2 ml) of a washed culture of *Sphingomonas* CHY-1 (Willison, 2004). Three types of control flasks were also prepared, which contained either unlabeled phenanthrene (added at 34 ppm as for SIP200), no additional carbon source, or sodium azide (7.7×10^{-5} mol/g dry soil; abiotic control). Microcosms containing [U- ^{13}C]-phenanthrene (SIP200 and SIP2000) were prepared in 9 replicates (triplicates at 3 time points), whereas controls with unlabelled phenanthrene or microcosms inoculated with strain CHY-1 were performed in 6 replicates (duplicates at 3 time points). Other control flasks were incubated in duplicates. Flasks were incubated at 25°C in static mode in a dark room.

PAH extraction from soil and quantification

Soil samples (1g) were mixed with 1g anhydrous sodium sulfate and 10 mL hexane in 30-mL glass tubes closed with Teflon-sealed screw-caps. Suspensions were homogenized by Vortex mixing and treatment in an ultrasonic bath for a total time of 1 h. Soil samples were extracted once more with hexane and organic phases were combined, centrifuged for 12 min at 7400 g before evaporation to dryness under argon. Dry extracts were dissolved in 0.5 ml acetonitrile. Residual ^{13}C -phenanthrene was extracted in the same way.

Quantification of the 16 priority PAHs was performed with a HP6890 gas chromatograph coupled to a HP5973 mass spectrometer (Agilent Technologies). PAHs were separated on a MDN12 column (30 m by 0.25 mm, 25 μm film thickness; Supelco) as previously described (Krivoboket al., 2003). The mass detector was operated in the single-ion monitoring mode. PAHs were eluted between 8.9 and 32.7 min. Concentrations were determined using calibration curves obtained from dilutions of a standard mixture of the 16 PAHs (0.1 mg/ml,

Agilent Technologies). ^{13}C -phenanthrene was quantified from the area of the peak detected at $m/z : 192$ using unlabelled phenanthrene as a standard.

Monitoring of mineralization by GC/MS analysis

Quantification of CO_2 evolved in the headspace of microcosms was performed by GC/MS analysis on 25 μl samples of the gas phase withdrawn with a gastight syringe. Analysis was carried out on the same apparatus as above equipped with an HP-PLOT/Q column (15 m by 0.32 mm, 20 μm film thickness; Agilent Technologies) with helium as carrier gas maintained at a flow rate of 36 ml/min. The GC was run in the isothermal mode at 40°C with a split ratio of 12:1. $^{13}\text{CO}_2$ ($m/z = 45$) or $^{12}\text{CO}_2$ ($m/z = 44$) were measured with the MS operated in the single-ion monitoring mode. Concentrations were determined from peak area using a calibration curve in the 0 to 200 μM range made with known mixtures of CO_2 in argon. Net $^{13}\text{CO}_2$ produced in microcosm headspaces was calculated by subtracting background $^{13}\text{CO}_2$ present in the air.

SSCP fingerprint analyses of 16S rRNA genes

For each tested experimental condition, 1g of soil (wet weight) was sampled and divided into three 250-mg soil aliquots, thus making 3 replicates per flask. DNA was extracted using the Power Soil extraction kit (MO BIO Laboratories). DNA samples were adjusted to 10 ng/ μl and used as templates to amplify the V3 region of the 16S rRNA genes. PCR conditions and subsequent capillary electrophoresis were performed as previously described (Zingeret al., 2007). SSCP profiles were computed and normalized. Nonmetric multidimensional scaling (NMDS) ordination was performed with the R software, using the vegan package (R Development Core Team, 2007). Environmental fitting was performed to evaluate the effects of time as a variable, and phenanthrene as a factor, on the bacterial community.

167

168 **DNA extraction and CsCl gradient fractionation**

169 For SIP200 and SIP2000 experiments, triplicate microcosms were sacrificed at day 5, 10 and
170 14. Soil samples (10 g) were transferred into 50-ml Falcon tubes and stored frozen at -80°C
171 until use. Triplicate 10-g samples of untreated soil collected at the beginning of the
172 experiment (day 0) served as controls. Duplicate soil samples were also collected at the
173 endpoint (day 14) from control microcosms with no addition. DNA was extracted using the
174 UltraClean Mega Soil DNA kit (MO BIO Laboratories) as recommended. DNA was eluted at
175 40-50 µg/ml in 8 mL.

176 For CsCl gradient fractionation, 2-ml portions of the DNA preparations were precipitated,
177 and then adjusted to 700 ng/µl in H₂O. Separation between ¹²C- and ¹³C-DNA was performed
178 by isopycnic ultracentrifugation on a CsCl gradient (Lueders et al., 2004). Gradients were
179 adjusted to an average density of 1.725 g/mL in 3.3 ml OptiSeal™ polyallomer centrifuge
180 tubes (Beckman Coulter), and loaded with 30 to 50 micrograms of soil DNA and 5 µL of
181 SYBR Safe™ (Invitrogen) as DNA stain (Martineau et al., 2008). For each run, a tube
182 containing equal amounts of ¹²C-DNA from *E. coli* and ¹³C-DNA *Sphingomonas* sp. CHY-1
183 (10 µg each) was processed as a means to control band separation and locate their position in
184 the gradient. ¹³C-labeled genomic DNA was prepared from *Sphingomonas* sp. CHY-1 grown
185 on ¹³C-succinate (Sigma-Aldrich) as sole carbon source. Preparation of genomic DNA from
186 this strain (Demaneche et al., 2004) and from *E. coli* strain DH5α (Ausubel et al., 1999)
187 followed published procedures. Ultracentrifugation was carried out at 413,000 x g_{AV} for 17 h
188 at 15°C, in a TLN-100 rotor using an Optima™ TLX Ultracentrifuge (Beckman Coulter).
189 Gradient fractionation was adapted from a published procedure (Manefield et al., 2002), using
190 a peristaltic pump operated at a flow rate of 0.18 ml/min and a Gilson fraction collector
191 equipped with a home-made device for holding centrifuge tubes. Twenty-two fractions, 150

192 μ l each, were obtained, from which DNA was precipitated according to (Pumphrey and
193 Madsen, 2008), using 1 μ l of 20 mg/ml glycogen as a carrier (Fermentas). Each fraction was
194 taken up in 20 μ l H₂O and DNA concentration was determined from UV absorbance
195 measurement with a ND-100 spectrophotometer (NanoDrop Technologies, Inc.). In plots of
196 DNA content versus fraction number, peak fractions of ¹³C-DNA and unlabeled DNA were 4
197 fractions apart.

199 **Construction of 16S rRNA gene libraries and DNA sequencing**

200 Both the labeled and unlabeled DNA isolated from the SIP200 and SIP2000 experiments, as
201 well as DNA extracted from untreated soil (control SIP 0D), was used as template to amplify
202 the nearly complete sequences of bacterial 16S rRNA genes. PCR amplification was
203 performed with primers 8F (AGAGTTTGATCCTGGCTCAG) and 1390R
204 (GACGGGCGGTGTGTACAA). PCR products were ligated into pCRTM4 TOPO[®] TA
205 vectors and electroporated into ElectroMAXTM DH10BTM T1 competent cells according to the
206 manufacturer's recommendations (Invitrogen). Bidirectional Sanger sequence reads were
207 obtained by standard procedures and assembled by PHRAP (<http://www.phrap.org>). Sequence
208 data obtained from clone libraries have been submitted to DDBJ/EMBL/GenBank under
209 accession numbers: FQ658499 to FQ660546 and FQ790244.

211 **Sequence analysis**

212 The 16S rRNA gene sequences were first affiliated to bacterial taxa using SeqMatch on the
213 Ribosomal Database Project (RDP) website (<http://rdp.cme.msu.edu/index.jsp> ; (Coleet al.,
214 2009)). Multiple sequence alignments and clustering into Operational Taxonomic Units
215 (OTUs) of the 2049 sequences considered herein were performed with mothur (Schlosset al.,
216 2009), using a 3% dissimilarity level between OTUs. Mothur was also used to generate

rarefaction curves and calculate richness estimators and diversity index (Table 1). A phylogenetic tree was generated using the TreeBuilder software on the RDP website.

Selection of 16S rRNA sequences representative of phenanthrene degraders

In DNA-SIP analysis, the set of 16S RNA sequences recovered from ^{13}C -DNA may be contaminated by unspecific sequences due to some overlap between labeled and unlabeled fractions after isopycnic centrifugation. A control experiment is usually carried out involving DNA analysis of a sample incubated with unlabeled substrate. After CsCl gradient separation, a fraction equivalent to that containing ^{13}C -DNA in the labeled experiment (heavy fraction), is chosen to run control PCR. In our hands, the choice of the heavy fraction for control purposes was somewhat arbitrary due to small changes in gradient density between samples. In addition, the reliability of this type of control requires that incubation conditions be identical between the SIP and control samples, which is difficult to achieve due to the inherent heterogeneity of soil. Hence, in his study, 16S rRNA sequences from ^{13}C -DNA libraries were considered representative of phenanthrene degraders if they complied with the following criteria : (i) they had to be part of OTUs represented by a minimum of 3 sequences per library (ii) they were not detected or poorly represented in libraries made from unlabeled DNA from the same run of centrifugation.

Real time PCR quantification of phylum-specific 16S rRNA genes

The copy number of 16S rRNA genes in soil DNA samples was estimated by quantitative PCR (qPCR) using universal or taxon-specific primers according to (Philippot et al., 2010). Amplification reactions were carried out in a StepOnePlus™ Real-Time PCR Systems (Applied Biosystems). Reaction mixtures contained 7.5 μl SYBRGreen® PCR Master Mix (Absolute QPCR SYBR Green Rox Abgene), 250 ng of T4 gene 32 (QBiogene), 4 ng of soil

DNA in a final volume of 15 μ l. Fluorescence acquisition was performed during 80°C steps to avoid interference of unspecific products. For each of the eleven 16S rRNA targets, a standard curve was established using serial dilutions of linearized plasmid pGEM-T (10^2 to 10^7 copies) containing a relevant 16S rRNA gene. Melting curves were generated after amplification by increasing the temperature from 80°C to 95°C. qPCR results are averages of three replicates, and are expressed as copy numbers per nanogram of DNA. The relative abundance of each taxon was calculated as the ratio of copy number of this taxon to the total number of 16S rRNA sequences, determined using universal primers.

RESULTS

Mineralization rates of ^{13}C -phenanthrene in microcosms

The soil used in this study had been exposed to chronic hydrocarbon contamination from road runoffs. It contained ca. 4.0 mg/kg of PAHs, including 0.156 ± 0.05 mg/kg of phenanthrene. For SIP experiments, fully labeled ^{13}C -phenanthrene was prepared and checked for purity and authenticity by GC-MS and NMR (supplementary Fig. S1). Experiments were carried out in microcosms containing 20 g of soil, and ^{13}C -phenanthrene supplied at 34 and 337 ppm, levels equivalent to approx. 200-fold (SIP200) or 2000-fold (SIP2000) the phenanthrene content of soil. Mineralization of the labeled C-source was monitored by GC-MS measurement of the $^{13}\text{CO}_2$ released in the headspace over 14 days (Fig. 1). In SIP2000 experiments, the $^{13}\text{CO}_2$ evolution reached a maximum rate after a 2-day lag phase, then leveled off after day 5. No lag phase was observed in microcosms inoculated with *Sphingomonas* CHY-1, a phenanthrene-degrading strain, suggesting that the delay reflected the time necessary for multiplication of soil PAH degraders. In SIP200 experiments, the rate of $^{13}\text{CO}_2$ was just above the background level detected in control microcosms, which received unlabelled phenanthrene or no addition. From the total amount of $^{13}\text{CO}_2$ released in the SIP2000 experiment, it was calculated that ca. 20% of the ^{13}C -phenanthrene had been mineralized. This value is most likely underestimated as an unknown amount of the carbon dioxide remained trapped in soil as bicarbonate. The residual labeled substrate in soil of the SIP2000 experiment amounted to 20.9 ± 2.3 ppm at day 5 (6.7 %) and 13.9 ± 1.0 ppm at day 14 (4.5 %), meaning that a major part of the added hydrocarbon had been degraded during the early stage of mineralization between days 2 and 5. Discrepancy between the mineralization rate and the extent of degradation of ^{13}C -phenanthrene (95%) might be explained in part by underestimations of either $^{13}\text{CO}_2$ (see above) or residual phenanthrene due to sequestration into soil particles (Johnsen et al., 2005).

Some of the labeled carbon was also incorporated into the organic matter of phenanthrene degraders.

Effect of phenanthrene on the overall bacterial community structure

To follow changes in the soil bacterial community upon incubation with phenanthrene, soil DNA was extracted at time intervals from SIP and control experiments, and 16S rRNA genes were first analyzed by SSCP fingerprinting. Electrophoresis profiles of PCR products targeting the V3 region were normalized and their distribution was analyzed by non metric multi-dimensional scaling (Fig. 2). Profiles were very similar suggesting that, at this level of observation, the bacterial community underwent little changes with the time of exposition (5, 10 or 14 d) and the dose of phenanthrene. Nevertheless, a clear trend emerged when comparing data sets at day 0, 5, 10 and 14, in that diversity profiles showed convergent time-dependent variations. On the other hand, diversity profiles obtained from phenanthrene-treated microcosms were more closely related to each other than to untreated controls, suggesting that phenanthrene-dependent shifts occurred in the bacterial population, but these shifts were little correlated to the concentration of phenanthrene.

The composition of the bacterial population in soil samples was then analyzed by real-time quantitative PCR (qPCR) using phylum-specific primers (class-specific for Proteobacteria), according to a method that has proven (Philippot, et al., 2010). Samples from the SIP2000 experiments showed, small time-dependent variations in 16S rRNA copy numbers (data not shown), suggesting that overall, the community structure was little affected by phenanthrene. However, when abundances were expressed as ratios with respect to the total copy numbers of 16S rRNA genes, a significant increase was found for the Beta- and Gammaproteobacteria (Fig. 3, grey bars). In contrast, Alphaproteobacteria and Actinobacteria showed unchanged or diminished proportions.

Betaproteobacteria enrichment in soil spiked with phenanthrene

Soil bacteria likely involved in phenanthrene degradation were identified based upon sequence analysis of 16S rRNA genes amplified from ^{13}C -DNA. Soil DNA samples recovered from SIP200 and SIP2000 experiments at day 5 and day 14 were separated into labeled and unlabeled fractions, both of which were used to prepare 16S rRNA gene libraries (Table 1). Sequences were affiliated to bacterial taxa using the RDP resources.. A detailed compilation of the 2049 sequences analyzed in this study is given in supplementary Table S1. As depicted in Fig. 4, the distribution of sequences among known bacterial phyla revealed that Proteobacteria were predominant in all libraries, the Beta class being the best represented. However, the proportion of Betaproteobacteria sequences was significantly higher in libraries made from labeled DNA, especially SIP2000 at day 5 (67%). In comparison, the library made out of unlabeled DNA (SIP2000 5D 12C) contained around 30% of Betaproteobacteria sequences, similar to the library of the untreated control (Fig. 4A and data not show). In the ^{13}C -DNA extracted from soil dosed with 10-fold less ^{13}C -phenanthrene, a Betaproteobacteria enrichment was also detectable, but only after 14 days (Fig. 4C).

Since the PCR-based method used to generate 16S rRNA sequence libraries might introduce biases in the determination of the actual proportions of bacterial taxa in soil, we implemented the quantitative PCR method described above to assess the copy number of taxon-specific 16S rRNA genes in ^{13}C -DNA fractions. Results illustrated in Fig. 3 showed that labeled DNA was specifically enriched in sequences affiliated to the Betaproteobacteria. The enrichment occurred within the first days of the incubation with ^{13}C -phenanthrene, and the proportion of Betaproteobacteria stayed above 10% throughout the 14-d incubation. Gammaproteobacteria were found to be significantly more abundant at day 10, suggesting that members of this class also accumulated at least transiently in response to phenanthrene. In comparison, Alphaproteobacteria, and Actinobacteria, as well as other taxa tested (data not shown) were

less represented and their copy number did not show a clear trend upon incubation with phenanthrene. Hence, consistent with the sequence analyses above, our quantitative data demonstrated that Betaproteobacteria became the dominant taxon in response to phenanthrene contamination of soil.

Identification of main PAH degraders

Further analysis of sequences in ^{13}C -libraries indicated that, at day 5, the community of PAH degraders exposed to 337 ppm phenanthrene was dominated by a few genera, including *Acidovorax*, *Rhodoferax*, *Hydrogenophaga* and *Polaromonas*, all members of the Comamonadaceae (Fig. 5A). Quite a few sequences affiliated to Rhodocyclaceae were also identified, but they were not all representative of phenanthrene degraders. Some of them were likely related to degraders as they belonged to OTUs only found in ^{13}C -libraries (OTU0, 1 and 17), while other were not because they belonged to OTUs mainly found in ^{12}C -libraries (OTU28, 51, 71; Fig. 6A). Hence, only some members of the Rhodocyclaceae would be able to degrade phenanthrene. A very similar pattern of dominant taxa was observed in the SIP200 experiment at day 14 (Fig. 5B), indicating that a 10-fold lower concentration of phenanthrene elicited the same soil population of degraders, although at a slower pace. Consistent with this idea, comparison of the SIP2000-5D-13C and SIP200-14D-13C libraries showed that they shared the highest number of common OTUs (78 of 161 or 48%, Fig S2), three of which were dominant in both libraries (OTUs 6, 4 and 17; Fig. 6A).

Apart from the Betaproteobacteria, two OTUs related to the Sphingomonadales were significantly represented in sequences obtained from ^{13}C -DNA, while almost inexistent in sequences retrieved from unlabeled DNA (OTUs 80 and 201; Fig. 6A). Sphingomonadales accounted for 32 to 37% of the Alphaproteobacteria sequences in SIP200 ^{13}C -libraries, and for 38 to 59% in SIP2000 ^{13}C -libraries, respectively (Fig. S3). One well-represented OTU

related to unclassified Gammaproteobacteria (OTU 2, 5% of the SIP2000-5D-13C library) was also detected in the set of sequences obtained from ^{13}C -DNA (Fig. 6A).

A phylogenetic tree was built with the best represented OTUs found in sequence libraries derived from ^{13}C -DNA (Fig. 6B). The analysis further highlighted that phenanthrene degraders were dominated by Betaproteobacteria. Except for OTU 4, OTU 6, OTU 12, OTU 14 and OTU 27, which were closely related to known isolates, most sequences were either associated to uncultured microorganisms or distantly related to known bacteria. In this respect, OTU 80 and OTU 201, affiliated to Sphingomonadaceae, showed sequences relatively distant from that of known PAH degraders in this bacterial family (Pinyakonget al., 2003; Demaneche, et al., 2004).

Diversity shifts in the PAH-degrading community as a function of the time of exposure to phenanthrene

Comparison of the SIP2000 sequence sets obtained from ^{13}C -DNA at 5 and 14 days showed that the proportion of Betaproteobacteria dropped from 67 to 35% (Fig. 4D and E). This change could largely be explained by a decline of the bacterial taxa that were identified as dominant PAH degraders at day 5 (Fig. 5A). At day 14, the best-represented Betaproteobacteria sequences were affiliated to *Thiobacillus*, a genus that has been seldom described for its ability to degrade aromatic hydrocarbons although it was recognized as a possible phenanthrene degrader (Bodouret al., 2003). A significant proportion of *Thiobacillus*-like sequences was also detected in the SIP200 sequence sets retrieved from ^{13}C -DNA. Similar to what was observed for the Betaproteobacteria, the OTU 2 related to the Gammaproteobacteria was detected in the SIP2000 library at day 5, but not at day 14.

On the other hand, Sphingomonads were detectable under all conditions tested, but their number tended to increase in the long run as exemplified by OTU 201, which was undetected at day 5 and represented by 11 sequences (50% of Alphaproteobacteria) at day 14 (Fig. 6A).

DISCUSSION

PAH pollution is persistent in various ecosystems including soils all around the world, and microbial biodegradation is considered the primary process responsible for its natural attenuation. This process has been extensively studied but for a rather limited range of culturable species (Peng, et al., 2008), and little is known on bacterial populations that mainly contribute to PAH removal in situ. In the present study, we have implemented a SIP approach and culture-independent methods to conduct a thorough investigation of phenanthrene degraders present in soil.

Overall, the soil community was dominated by Proteobacteria, consistent with previous reports documenting the prevalence of this phylum in different soils (Roeschet al., 2007). Moreover, our results brought convergent pieces of evidence that Betaproteobacteria play a major role in the degradation of phenanthrene in soil. This is supported by the increased proportion of ribosomal sequences representative of this class in ¹³C-DNA in response to phenanthrene, as shown by both qPCR and sequence analysis of cloned 16S rRNA genes.

The main PAH degraders identified in this work mostly belong to the Burkholderiales, with *Acidovorax*, *Rhodferax*, *Hydrogenophaga* and *Polaromonas* as the best represented genera. Likewise, a SIP study targeting naphthalene degraders, revealed that *Polaromonas*, *Rhodferax* and *Acidovorax* mainly contribute to in situ degradation (Jeon, et al., 2003), suggesting that the same type of bacteria are responsible for the biodegradation of naphthalene and phenanthrene in soil. Based on 16S rRNA sequence comparison, the dominant *Acidovorax* identified in this study strikingly resembled members of the same genus

previously identified in a bioreactor treating a PAH-contaminated soil (Singleton, et al., 2005). A strain isolated from this bioreactor, *Acidovorax* NA3, has been recently described as a new PAH degrader (Singleton, et al., 2009).

Interestingly, most PAH degraders identified in the present study are related to poorly described taxa, consistent with the fact that a majority of soil bacteria are unknown. They differ from PAH-degrading isolates studied so far, and even members of well-known degraders such as Sphingomonadaceae (Demaneche, et al., 2004; Leys, et al., 2004), were found to be distantly related to described strains (Fig. 6). Gammaproteobacteria are represented by one OTU unrelated to known genera of this class, such as *Pseudomonas* (OTU2; Fig. 6), but very similar to a pyrene-degrading uncultured bacterium detected by SIP in a bioreactor (Singleton, et al., 2006). Moreover, our survey highlighted bacterial taxa affiliated to Rhodocyclaceae and *Thiobacillus*, which appear as new PAH degraders. Among Rhodocyclaceae, denitrifying bacteria belonging to the *Azoarcus*, *Aromatoleum*, *Denitratisoma* and *Thauera* genera were shown to anaerobically degrade phenolic compounds (Sueoka et al., 2009) and alkylbenzenes (Rabus and Widdel, 1995; Rotaru et al., 2010), but no Rhodocyclaceae member endowed with PAH-degrading ability has yet been described.

Similar populations of degraders were found upon soil treatment with 10-fold different levels of phenanthrene. This observation indicated that, at the highest concentration tested, no adverse effect could be noticed on any of the bacterial taxa involved in phenanthrene degradation. In contrast, different naphthalene degraders were identified in a ground aquifer, depending on the concentration of the hydrocarbon (Huanget al., 2009). In the latter case, the toxic or inhibitory effect of naphthalene might have influenced bacterial selection, in accordance with previous observations (Jeon, et al., 2003).

Time-dependent changes in the population of degraders were observed upon incubation with phenanthrene. The rapid phase of phenanthrene mineralization during the first days coincided with the multiplication in soil of the main degraders discussed above. In the SIP2000 experiment, dominant degraders at day 5 drastically declined at day 14. During this time period, the soil bacterial community underwent little change as shown by SSCP and qPCR analyses. Hence, the decline appeared to specifically affect the subpopulation of PAH degraders, maybe because phenanthrene became less bioavailable. Although significant amounts of residual phenanthrene were detected in microcosms after 5 and 14 days, the time course of mineralization suggested that it became limiting at day 5, perhaps due to its sequestration or sorption on soil particles. In the SIP200 experiment, the lower dose of phenanthrene likely elicited slower growth, and might have sustained PAH degraders for a longer time. Hence, our results provide evidence that the subpopulation of degraders undergo relatively rapid dynamic changes in response to the level of PAHs available in soil.

Acknowledgments

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546 **Table 1 : Description of the nine 16S rRNA sequence libraries considered in the DNA SIP experiments.**

547

Library	Sequence	OTU	Richness estimation		Diversity Index
	number	number	Chao1	Ace	Shannon
SIP1 0D	230	173	468	892	4.87
SIP200 5D 13C	167	132	810	783	4.67
SIP200 5D 12C	147	135	938	1897	4.82
SIP200 14D 13C	353	161	748	2144	4.32
SIP200 14D 12C	259	233	572	956	4.96
SIP2000 5D 13C	423	173	536	942	3.89
SIP2000 5D 12C	228	186	739	1981	5.01
SIP2000 14D 13C	212	135	476	877	4.20
SIP2000 14D 12C	28	27	118	182	3.23

Legends to figures

Figure 1: Time course of $^{13}\text{CO}_2$ evolution during SIP experiments as measured by GC/MS. $^{13}\text{CO}_2$ was measured in the headspace of microcosms supplied with either 34 ppm (Δ ; SIP200) or 337 ppm (\blacktriangle ; SIP2000) ^{13}C -phenanthrene, at the beginning of the incubation. Two microcosms spiked with 337 ppm ^{13}C -phenanthrene were inoculated with *Sphingomonas* CHY-1 (\blacklozenge). Other experimental conditions included addition of 34 ppm unlabelled phenanthrene (\square), no addition (\blacksquare) and abiotic control (\circ). Data show net $^{13}\text{CO}_2$ evolution calculated by subtracting natural $^{13}\text{CO}_2$ present in the air. Data are averages of three replicate treatments for SIP200 and SIP2000, and two replicates for other experimental conditions. Error bars indicate standard deviations.

Figure 2: NMDS ordination of bacterial communities exposed to phenanthrene, based on SSCP fingerprint analysis.

SSCP profiles were computed and compared through nonmetric multidimensional scaling using a stress value of 9.46. Data fitting was applied to examine the effects of phenanthrene and incubation time on the ordination. Phenanthrene concentration was 34 ppm (\blacksquare), 337 ppm (\blacktriangle) or background level (\bullet). Incubation time (days) was 0 (orange symbols), 5 (red), 10 (blue), or 14 (green). Vector indicates time variable ($P < 0.001$), centroides denote phenanthrene level added: none (PheNo), 34 ppm (PheLw) or 337 ppm (PheHg) ($P < 0.002$).

Figure 3: Relative abundances of phylum-specific 16S rRNA genes in soil DNA during incubation with ^{13}C -phenanthrene. Template was either total soil DNA (grey bars) or ^{13}C -DNA (black bars) extracted from SIP2000 experiments at 5, 10 and 14 days (5D, 10D and 14D, respectively). A control DNA sample was obtained from soil incubated for 14 days

without phenanthrene (T14D). Stars denote significant differences compared to control DNA from non-incubated untreated soil (0D ; $P < 0.05$). A: Alphaproteobacteria; B: Betaproteobacteria; C: Gammaproteobacteria; D: Actinobacteria.

Figure 4: Prevalence of Betaproteobacteria-specific sequences in 16S rRNA gene libraries recovered from ^{13}C -labeled soil DNA.

Chart pies represent sequence repartition into main taxa for the following 16S rRNA gene libraries: A, SIP1 0D (control); B, SIP200 5D ^{13}C ; C, SIP200 14D ^{13}C ; D, SIP2000 5D ^{13}C ; E, SIP2000 14D ^{13}C . Other phyla include Bacteroidetes, Chloroflexi, Spirochete, Verrucomicrobia, Cyanobacteria, Gemmatimonadetes, Firmicutes, Planctomycete and Nitrospira.

Figure 5 : Distribution of Betaproteobacteria into best-represented families and genera.

Sequences were from ^{13}C -DNA libraries prepared at day 5 and 14 from the SIP2000 (A) and the SIP200 (B) experiments, or from a control DNA library made from untreated soil (SIP 0D). Ratios were calculated as the number of sequences per taxon versus the total number of sequences affiliated to Betaproteobacteria in each library.

Figure 6: Major bacterial taxa likely involved in ^{13}C -phenanthrene degradation.

From a compilation of all 2049 16S rRNA gene sequences considered herein, grouping in OTUs was performed with mothur. A : Diagram showing the sequence number of those OTUs exclusively or mainly consisting of sequences derived from ^{13}C -DNA. B : Phylogenetic tree illustrating the relationships between these OTUs and most similar sequences found in the RDP data-base. Affiliation to relevant bacterial taxa is indicated on the left. The tree was

597 built with TreeBuilder found on the RDP website, using the *Aquifex pyrophilus* 16S rRNA
598 gene as an outgroup sequence.

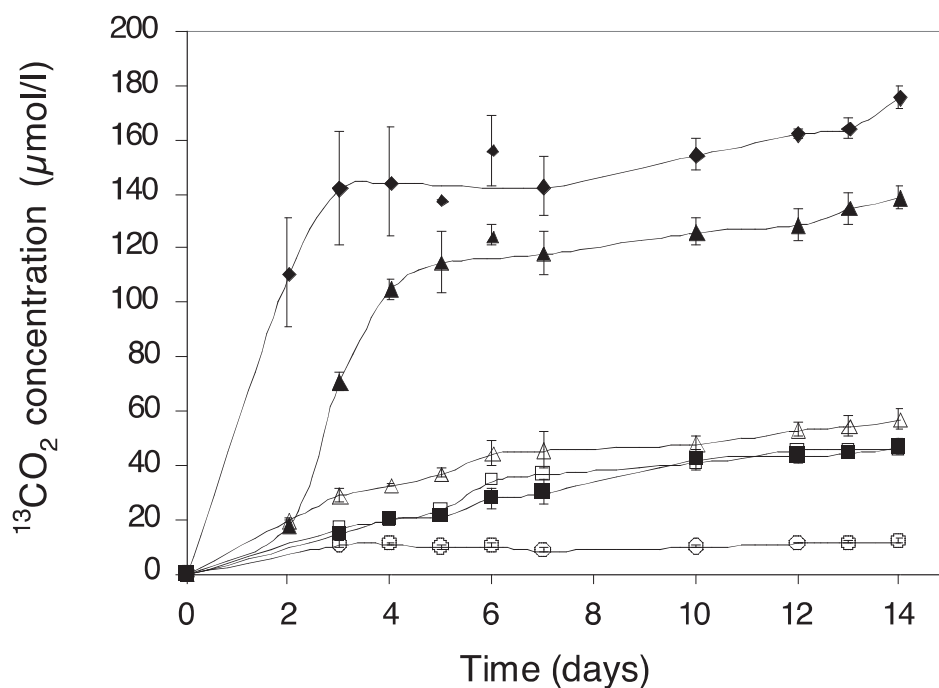


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Figure

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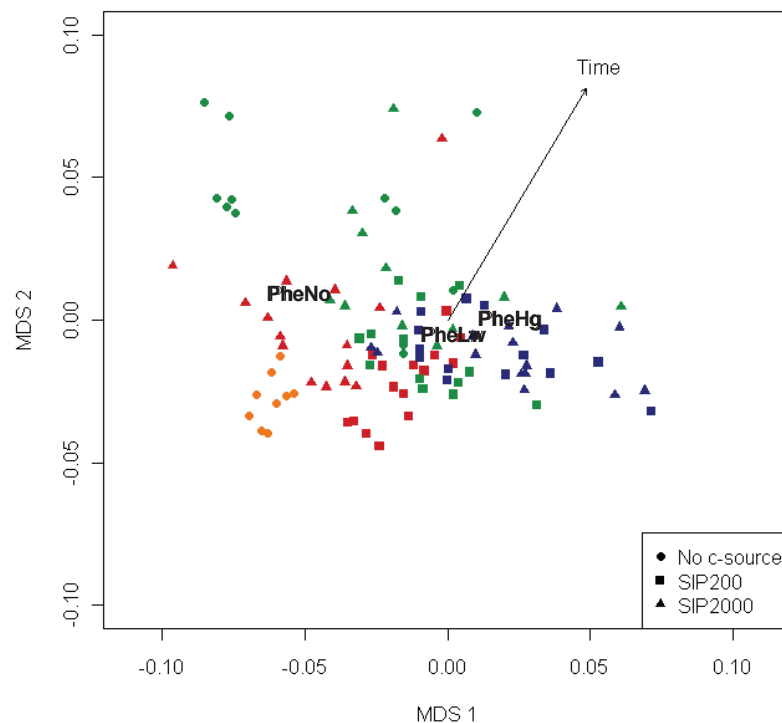


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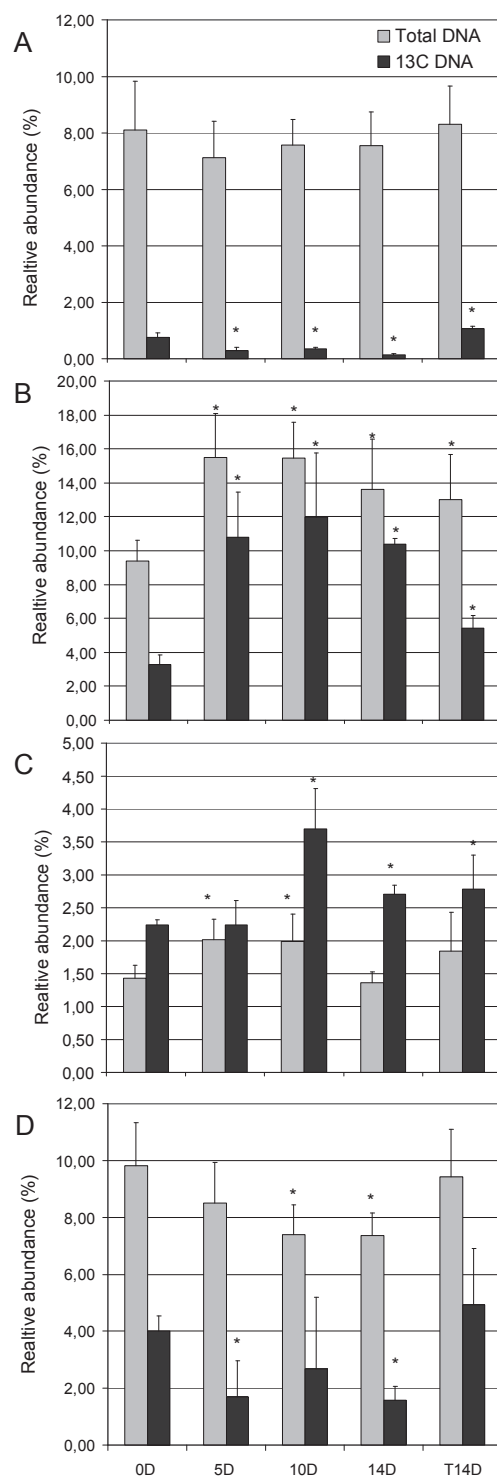


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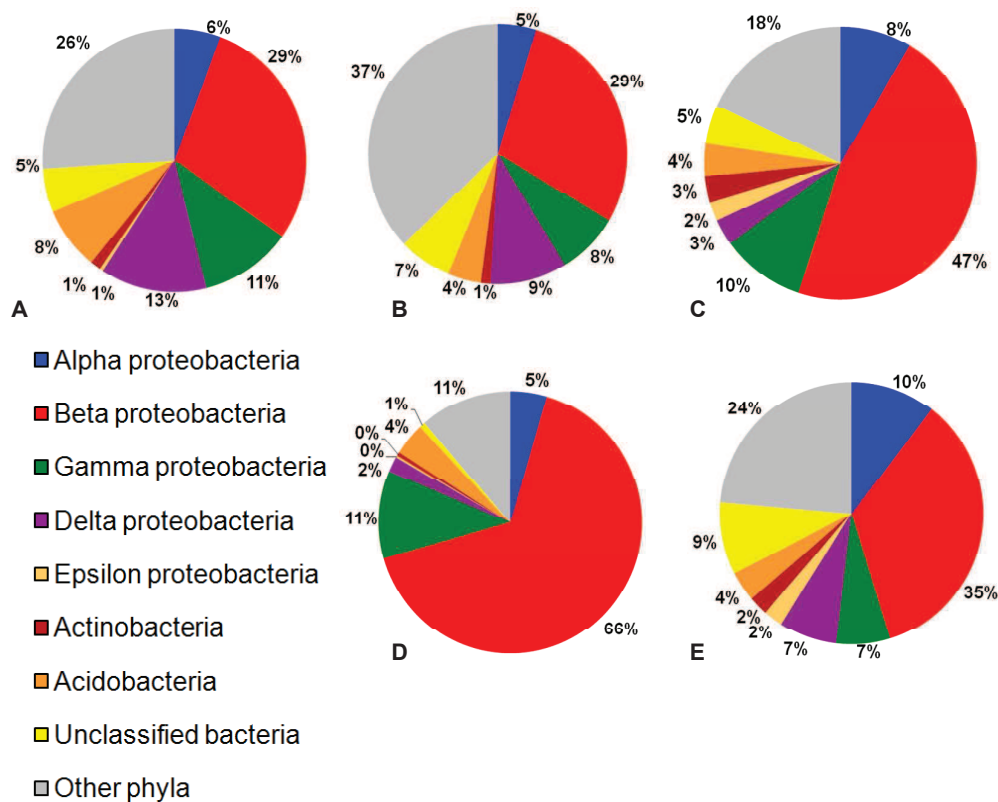


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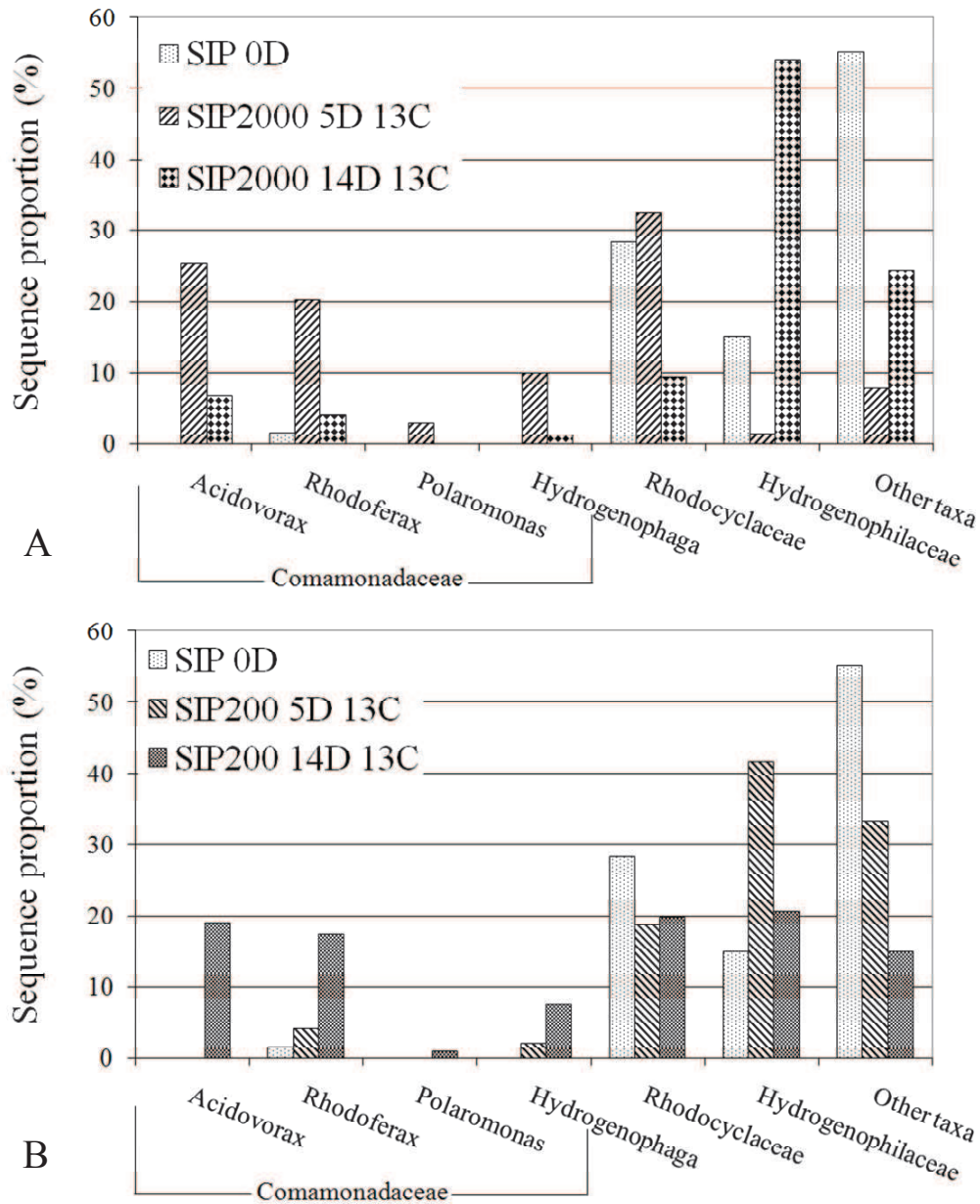


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Figure
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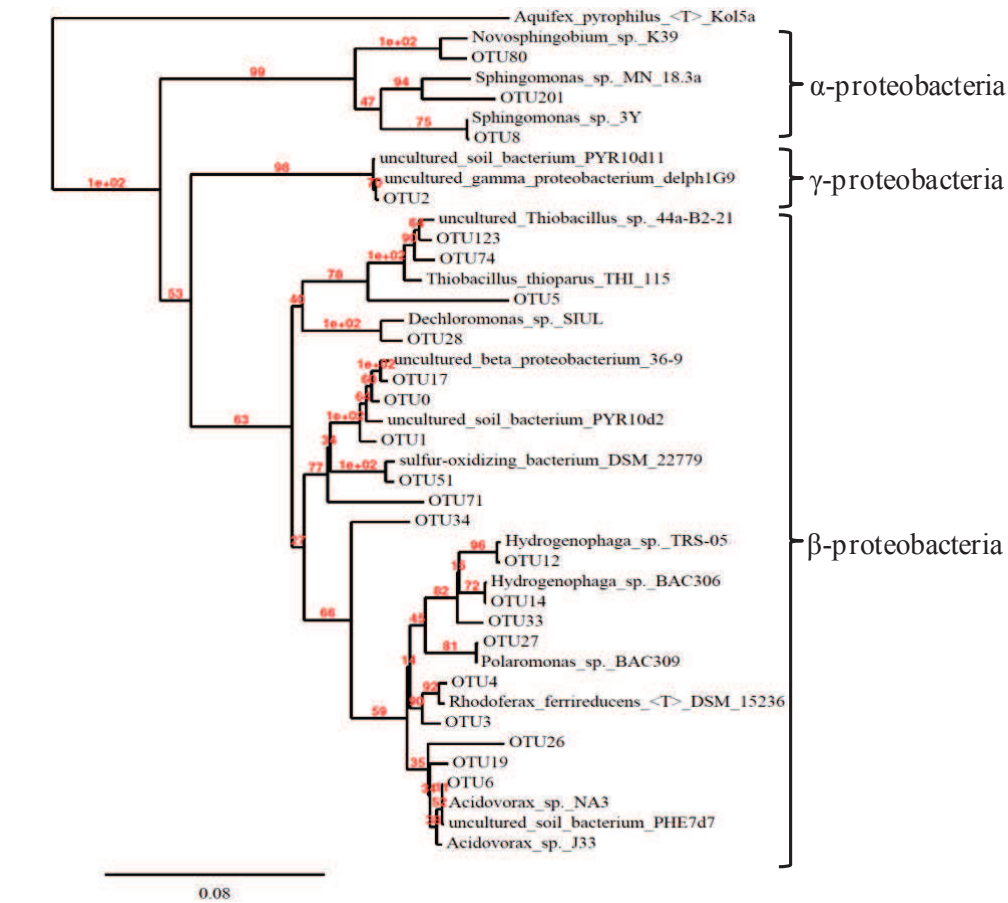
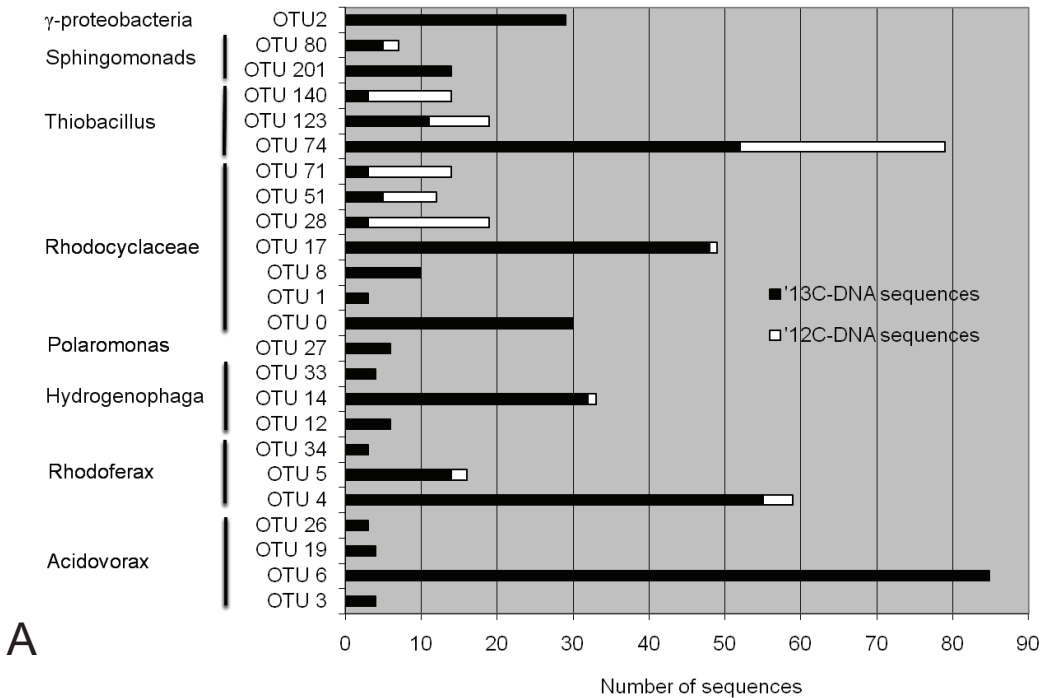


Figure 6

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